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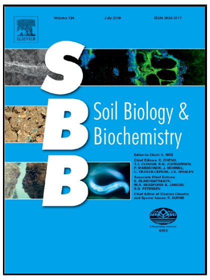

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Immediate and carry-over effects of increased soil frost on soil respiration and microbial activity in a spruce forest

Kaijun Yang[1, 2], Changhui Peng[3, 4], Josep Peñuelas[2,5], Paul Kardol[6], Zhijie Li[1, 7], Li Zhang[1], Xiangyin Ni[1], Kai Yue[1], Bo Tan[1], Rui Yin[8], Zhenfeng Xu[1]*

1. Institute of Ecology and Forest, Sichuan Agricultural University, Chengdu, China
2. Global Ecology Unit CREAF-CSIC-UAB, CSIC, Barcelona, Catalonia, Spain
3. Department of Biological Science, Institute of Environment Sciences, University of Quebec at Montreal, Montreal, Canada;
4. Center for Ecological Forecasting and Global Change, College of Forestry, Northwest Agriculture & Forest University, Yangling, Shaanxi, China;
5. CREAF, Barcelona, Spain
6. Department of Forest Ecology and Management, Swedish University of Agricultural Science, 90183, Umeå, Sweden
7. Forschungszentrum Jülich GmbH, Agrosphere (IBG-3), Jülich, Germany
8. Helmholtz-Centre for Environmental Research-UFZ, Department of Community Ecology, Theodor-Lieser-Strasse 4, 06110 Halle (Saale), Germany

Correspondence: Dr. Zhenfeng Xu, Email: xuzf@sicau.edu.cn, Tel: +86 28 86290957, No.211, Huimin Road, Wenjiang, Chengdu, Sichuan, 611130 China

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Abstract

Increased soil frost associated with winter climate change could have immediate and carry-over effects on biological processes in high-altitude forest soils, but the nature of these processes remain poorly understood. We conducted a snow-exclusion experiment to investigate the immediate and cross-seasonal effects of increased soil frost on soil CO_2 efflux and biological activity in a subalpine spruce forest on the eastern Tibetan Plateau, China. The increased frost reduced soil CO_2 efflux by ~15 and ~19% in the winters of 2015/2016 and 2016/2017, respectively. Increased frost also tended to decrease soil basal respiration, the amount of microbial phospholipid fatty acids and the activities of enzymes involved in soil carbon cycling during the winters. Winter soil nitrogen availabilities were higher in the snow-exclusion treatment than in the control plots. However, these effects did not carry over to the following growing season. Our results suggest that increased frost reduces winter soil respiration by direct environmental effects (e.g. soil temperature) and indirect biological processes (e.g. microbial biomass and activity), whereas increased frost did not induce any cross-seasonal effects. These findings underscore the ecological importance of seasonal snowpack and microbe-associated carbon processes in subalpine forests where winter snowfall is decreasing substantially.

1. Introduction

Seasonal snow cover is a major control of biogeochemical cycling in cold environments (Jusselme et al., 2016). Many snowy areas at high latitudes and altitudes have experienced substantial climate change in recent decades, and this trend is predicted to continue in this century (IPCC, 2013). Climate-induced changes are particularly rapid in high latitude and alpine ecosystems, where rising temperatures have profound effects on winter conditions, such as snowfall, soil frost and extreme climatic events (Liu et al., 2012; IPCC, 2013). Winter precipitation in these regions is more likely to occur in the form of rain rather than snow due to winter warming (Wang et al., 2016). The lack of insulating snow cover could consequently increase soil frost (Groffman et al., 2001a; Bokhorst et al., 2013), which could in turn have complex and large impacts on soil microbiological and biochemical processes in cold forests.

Winter biological processes and their controls are not as well understood as growing-season processes, despite the importance of winter warming and biological activity in cold systems (Sanders-DeMott and Templer, 2017). Recent studies have found that soil biological processes are sensitive to warming-induced changes in winter conditions, especially snow cover and soil frost (Haei and Laudon, 2015; De Long et al. 2016; Li et al., 2016). Snow removal has negative or neutral influences on winter soil respiration in boreal and temperate forests (Groffman et al., 2006; Aanderud et al., 2013; Bokhorst et al., 2013), but changes in snow cover can also alter biological processes in snow-free periods (Muhr et al., 2009; Wubs et al., 2018). Snow exclusion can suppress soil respiration in the snow-free season in high-latitude ecosystems (Öquist and Laudon, 2008; Zhao et al. 2017). To our knowledge, however, soil biological responses to changing soil frost have rarely been investigated in both snow-covered and snow-free periods in the same experiment. A better understanding of the impacts of intensified soil frost on the biotic and abiotic controls over the dynamics of soil C in both snow-covered and snow-free seasons is thus essential for accurately modeling and predicting potential C feedbacks in a warmer world.

Altered soil frost may directly and indirectly affect soil C cycling, such as by affecting soil temperature and moisture (Aanderud et al., 2013; Song et al., 2017), soil microbial biomass and activity (Monson et al., 2006; Sorensen et al., 2016) and substrate quality and quantity (Brooks et al., 2004; Steinweg et al., 2008; Comerford et al., 2013). The direction and magnitude of biological responses to increased frost may be determined by the combined effect of these processes. Diverse techniques have provided insight in recent years into the impacts of winter climate change on soil C cycling as the importance of winter processes has increased (Li et al., 2016). Most field-manipulation studies have focused mainly on high-latitude systems, including peatlands and boreal forests (Sanders-DeMott and Templer, 2017). Soil biological responses from low-latitude cold systems with unique winter conditions, such as Tibetan subalpine forests, however, remain unknown.

The Tibetan Plateau, the Earth's 'Third Pole', has warmed substantially, especially in winter (Chen et al., 2013). Winter snowfall has decreased at a rate of 0.6 mm y^{-1} in recent decades (Wang et al., 2016; Xu et al., 2017). Seasonal snowpack in this region has unique characteristics, such as shorter duration and shallower depth relative to high latitudes. Winter soil temperature is also near the physical melting point and is sensitive to changes in snow cover (Li et al., 2017). The subalpine forests of southwestern China contain a large amount of soil organic C (Zhang et al., 2013), but most studies of global-change biology have only focused on responses during the growing season (e.g. Xu et al., 2012; 2015; Yin et al., 2013), even though warming is extremely pronounced and microbial activity is unexpectedly high during winter (Wang et al., 2016; Wang et al. 2012; Tan et al., 2014). Future soil frost will also likely affect the biological and environmental controls of soil C cycling in these forests, but the underlying mechanisms of such processes remain unknown.

We conducted a snow-manipulation experiment to investigate the immediate and carry-over effects of increased winter frost on soil C cycling in a spruce forest on the eastern Tibetan Plateau. Specifically, we hypothesized that (1) More intense frost in the soil as a result of exclusion of snow would decrease microbial activity and soil respiration in winter; (2) frost-reduced biological processes would carry over into the subsequent snow-free growing season.

2. Materials and methods

2.1 Site description

The field manipulation experiment was conducted in a dragon spruce (*Picea asperata* Mast.) stand at the Long-term Research Station of Alpine Forest Ecosystems of Sichuan Agricultural University on the eastern Tibetan Plateau of China (31°15′N, 102°53′E; 3021 m a.s.l.). The mean annual precipitation and temperature are 850 mm and 3.0 °C, respectively. Snow generally begins to accumulate in late November and melts in late March the following year. The soil is classified as a Cambic Umbrisol (IUSS Working Group WRB, 2007). The soil (0-15 cm) contains 88.5 g kg^{-1} organic C and 5.4 g kg^{-1} nitrogen (N) and has a pH of 6.4 (Li et al., 2017).

2.2 Experimental design

Winter snowfall was excluded using shelters to intensify soil frost. Shelters are considered to be a useful tool for studying the responses of soil processes to winter climate change because they can effectively reduce snow cover and minimize the changes in other unwanted environmental conditions (Li et al., 2016). In early November 2015, six wooden roofs were set up in the spruce forest to prevent the accumulation of snow on the ground. One control plot was established in the vicinity of each roof. The roofs were 2 m in height with a ground area of 3 × 3 m. The snow manipulation began in mid-November and ended in late March the following year.

2.3 Soil sampling

Soil samples were collected from the topsoil (0-15 cm) in the frost period (FP, late January), early thawing period (ETP, early April) and the middle of the growing season

(MGS, mid-August) in the year of 2016 and 2017, respectively. Three cores (5 cm in diameter, 15 cm in depth) were collected in each plot at each sampling. The three cores from each plot were combined to form one composite sample. Each composite sample was passed through a 2-mm sieve, and any visible living plant material was manually removed. The sieved soil was used for biochemical analysis.

2.4 Soil CO_2 efflux

Two PVC collars (20 cm in diameter, 12 cm in height) were permanently installed in each plot for measuring soil respiration. Soil CO_2 efflux was measured using a portable infrared gas analyzer (Li-8100, Li-Cor Inc., Lincoln, USA) between 10:00 and 14:00 (Beijing time, China Standard Time) approximately every two weeks during the experimental period. Simultaneously, soil temperature and volumetric moisture at a depth of 5 cm were measured nearby each collar using an auxiliary soil temperature probe (Omega Engineering Inc., USA) and a Theta probe (Delta-T Devices, Cambridge, UK), respectively. Small red flags were attached to the PVC collars in the control plots to minimize disturbance during the period of snow cover. During the winters of 2015/2016 (four times) and 2016/2017 (once), the surface snow was removed carefully from the top of the collars when the snowpack was thicker than the height of the collar. We then waited 5 min to allow the system to equilibrate before measuring the CO_2 efflux. The removed snow was gently backfilled after the measurements. We measured CO_2 efflux under the natural snowpack (~10 cm) in the winter of 2015/2016 in the same forest stand adjacent to the snow manipulation site. CO_2 efflux was also measured after removing the snow within and around the collars. CO_2 efflux did not differ significantly before and after snow removal (unpublished data). Snow removal therefore likely negligibly affected the quantification of immediate CO_2 efflux, at least within an interval of a few minutes.

2.5 Microclimate, extractable N and microbial respiration

Air temperature 2 m above the ground in the forest stand was measured using Thermochron iButton DS1923–F5 Recorders (Maxim Dallas Semiconductor Corp., USA) every 2 h during the experimental period. Meanwhile, soil temperatures 5 cm below the surface were recorded in the snow-exclusion and control plots, respectively. Snow depth in the control plots was measured by a metal ruler approximately every two weeks during winter.

Soil extractable N (nitrate, NO_3^--N, and ammonium, NH_4^+-N) was extracted with 2 M KCl (1:5 soil:solution). The extracts were shaken for 1 h and filtered with a filter paper. The concentrations of NO_3^--N and NH_4^+-N in the extracts were determined by colorimetry (Li et al., 2017).

The rate of soil microbial respiration was estimated using alkali absorption (Anderson et al., 1982). Soil samples (50 g) were incubated in 1-L jars at 20 °C for 2 weeks. Empty jars without soil were used as controls. The CO_2 produced was captured with 0.5 M NaOH in a beaker suspended inside each jar. The NaOH solution was removed and titrated with 0.25 M HCl solution to determine the amount of CO_2 produced. Microbial respiration was reported as mg CO_2-C kg^{-1} soil d^{-1}.

2.6 Aggregate fraction and fine-root biomass

Aggregates were isolated as described by Kristiansen et al. (2006). Two soil cores from each plot were collected from the 0-15 cm layer using an auger 10 cm in diameter in the early thawing periods of 2015/2016 and 2016/2017 winters. Soil samples were air-dried to optimal moisture (~10-15%) that would allow limited mechanical stress to maximize brittle failure along natural planes of weakness, and the samples were then gently manually crumbled to <8 mm. The recovered samples were transferred to a nest of sieves (2 and 0.25 mm) and shaken at 100 min^{-1} for 2 min. All visible roots and stones were removed, and aggregates >2 mm (large macroaggregates) were collected. The same procedure was used for the material retained on the 0.25 mm sieve, isolating an aggregate size class 0.25-2 mm (small macroaggregates). The remaining material passing through the 0.25 mm sieve was identified as aggregate class <0.25 mm (microaggregates).

Two soil cores were collected from each plot using an auger (15 cm long and 10 cm in diameter) in the ETPs of 2015/2016 and 2016/2017 winters. Root samples were washed in the laboratory on sieves (mesh size 0.1 mm) and dried to constant weight at 65 °C. Fine roots (<2 mm in diameter) were separated into live and dead components based on their color and mechanical consistency.

2.7 Assays of soil phospholipid fatty acids and enzyme activities

Microbial biomass was estimated as the total extractable phospholipid fatty acids (PLFAs) with a modified method described by White et al. (1996). Lipids from 2 g of fresh soil were extracted in a chloroform-methanol-phosphate buffer mixture (1:2:0.8). The phospholipids in the extracts were transformed by alkaline methanolysis into fatty acid methyl esters (FAMEs), which were identified by gas chromatography/mass spectrometry (GC/MS-QP2010 Series, Shimadzu, Japan). Fatty acids were quantified by comparisons of the peak areas from the sample with the peak areas of internal standards at 19:0 (nonadecanoic methyl ester) of the known concentration. The areas were used to estimate the abundance of PLFA markers, which were expressed as nmole g^{-1} dry soil.

We assessed the activities of four enzymes involved in soil C cycling: two hydrolytic enzymes, β-glucosidase (BG) that catalyzes one of the later steps of cellulose degradation and β-N-acetyl-glucosaminidase (NAG) involved in the breakdown of chitin and fungal cell walls, and two oxidases, polyphenol oxidase (PPO) that breaks down recalcitrant polymers such as lignin and humic compounds and peroxidase (POD), a nonspecific enzyme that oxidizes and depolymerizes lignin. The activities were measured using assay techniques described by Allison and Jastrow (2006). Substrate solutions were 5 mM pNP-β-glucopyranoside for BG, 50 mM pyrogallol and 50 mM EDTA for PPO, 2 mM pNP-β-N-acetylglucosaminide for NAG and 5 mM L-DOPA and 10 μL of 0.3% H_2O_2 for POD. Activities were determined using a microplate spectrophotometer and expressed as μmol of substrate produced or consumed h^{-1} g^{-1} dry soil.

2.8 Data analysis

A repeated-measures ANOVA was used to test the effects of treatment, sampling date and their interactions on all response variables. A Bonferroni post hoc test was used to examine the treatment effect on the variables on a given sampling date when the interaction of treatment and sampling date was significant ($P < 0.05$), and a paired *t*-test was used when the interaction was not significant. All data were tested for the assumptions of an ANOVA before analysis. Heterogeneous data were ln-transformed before analysis. An exponential regression model was used to describe the relationship between CO_2 efflux and soil temperature during specific periods (winter, growing season and entire year). All data from two winters or growing seasons were used for the analyses due to the limited number of measurements. Winter was defined as the period between the first day in autumn and the last day in spring when soil temperature was continually below 5 °C for 5 d in the control plots. The temperature sensitivity (Q_{10}) of soil respiration was estimated using van't Hoff equation (Van's Hoff, 1898). $R = \alpha \times e^{\beta \times T}$, Where R is the soil respiration rate (μmol m^{-2} s^{-1}), T is the soil temperature at 5 cm (°C), α and β are parameters. The Q_{10} values are calculated as: $Q_{10} = e^{10 \times \beta}$. All statistical tests were performed using the Software Statistical Package for the Social Sciences (SPSS) version 17.0 (IBM SPSS Statistics Inc., Chicago, IL, USA).

3. Results

3.1 Treatment effect on winter soil conditions

The mean and minimum air temperatures were -2.1 and -14.1 °C during the winter of 2015/2016 and -0.9 °C and -6.4 °C during the winter of 2016/2017, respectively (Figure 1 a). The maximum snow depth was 40 cm in the winter of 2015/2016 but only 23 cm in the winter of 2016/2017. The mean air temperature in the winter of 2015/2016 (-2.1 °C) was comparable to the seven-year average of -2.4 °C for 2010-2016. The mean air temperature in the winter of 2016/2017 (-0.9 °C), however, was the highest in the last seven winters and 1.5 °C higher than the mean.

The snow-exclusion treatment successfully created a more intense frost regime in both winters (Figure 1 a). The minimum daily mean soil temperatures were -2.2 °C (2015/2016) and -2.4 °C (2016/2017) in the snow-exclusion plots but were only -0.5 °C (2015/2016) and -1.3 °C (2016/2017) in the control plots. The numbers of days with differences in soil temperature ≥0.5 °C between the treatment and control plots were 42 and 56 for the winters of 2015/2016 and 2016/2017, respectively. Such differences were mainly during mid- and late winter when snow cover was >10 cm. Soil temperature fluctuated more in the treatment than the control plots. Volumetric soil moisture was similar between the treatment and control plots across the two years (F=3.364, P = 0.116, Figure 1 b).

3.2 Soil CO_2 efflux

The snow exclusion lowered CO_2 efflux early in the winter of 2015/2016 and in mid-winter of 2016/2017. The snow exclusion reduced CO_2 efflux by averages of 15% and 19% in the winters of 2015/2016 and 2016/2017, respectively, and these reductions were statistically significant (F = 11.13, P < 0.01 for the winter of 2015/2016; F = 9.143, P < 0.05 for the winter of 2016/2017). The snow-exclusion manipulation, however, did not affect CO_2 efflux during the snow-free growing seasons (F = 1.065, P = 0.323 for 2016; F = 1.354, P =

0.305 for 2017). Mean CO_2 efflux differed marginally between the frost regimes in the winter of 2015/2016 ($t = 2.006$, $P = 0.076$; Table 1), but differed significantly between the regimes in the winter of 2016/2017 ($t = 3.909$, $P < 0.01$). Mean CO_2 efflux nevertheless did not differ significantly between the treatment and control plots in either growing season ($t = -1.584$, $P = 0.335$ for 2016; $t = -0.285$, $P = 0.465$ for 2017).

Soil CO_2 efflux increased exponentially with soil temperature throughout the study period (Figure 3a-c). Soil temperature explained 82-83% of the variation in CO_2 efflux during the growing seasons (Figure 3b) but explained only 52-53% of the variations in the winters (Figure 3a). Soil temperature explained 90-91% of the variance in CO_2 effluxes when the data for the two years were pooled (Figure 3c). The temperature sensitivity (Q_{10}) of the CO_2 efflux was 23.3, 3.2 and 4.4 in the snow-exclusion treatment plots and 22.6, 3.3 and 4.7 in the control plots for winter, growing season and the entire year, respectively. Q_{10} did not differ significantly between the frost regimes for each period modeled (all $P > 0.05$).

3.3 Soil PLFAs and microbial respiration

The intensified frost tended to decrease the soil PLFAs biomarkers. PLFA content was lower in the snow-exclusion treatment than in the control plots in the FPs of 2015/2016 ($t = -2.072$, $P < 0.05$) and 2016/2017 ($t = -3.686$, $P < 0.05$; Figure 4) but did not differ significantly in the MGSs of 2016 ($t = 1.368$, $P = 0.245$) or 2017 ($t = 0.035$, $P = 0.895$).

Microbial activity, measured as basal respiration without roots, was estimated by determining CO_2 emission. The intensified frost tended to decrease soil microbial respiration in the winter. The snow-exclusion treatment negatively affected soil microbial respiration in the FP of 2015/2016 ($t = -0.918$, $P < 0.05$; Figure 5) and in the ETP of 2016/2017 ($t = -5.821$, $P < 0.01$) but had no effect in the MGSs of 2016 and 2017 (both $P > 0.05$)

3.4 Soil enzymes

The activities of the soil enzymes varied significantly with sampling date (all $P < 0.01$,

Figure 6a-d). The snow-exclusion treatment tended to reduce the enzyme activities in the winter. Activity was significantly lower in the treatment than in the control plots for BG in the ETP of 2015/2016 (t = -1.975, $P < 0.05$; Figure 6a) and for PPO in the ETP of 2016/2017 (t = -2.643, $P < 0.05$; Figure 6b). The intensified frost decreased POD activity in the FPs of 2016 and 2017 (both $P < 0.05$, Figure 6c) and decreased NAG activity in the ETPs of 2016 and 2017 (both $P < 0.05$, Figure 6d) but did not significantly affect the activities of the enzymes in the MGSs of 2016 or 2017.

3.5 Soil extractable N

Frost treatment, sampling date and their interaction all significantly affected soil NH_4^+-N concentration (all $P < 0.05$, Figure 7a). The snow-exclusion treatment increased NH_4^+-N concentrations in the FP and ETP of 2015/2016 (all $P < 0.01$) but not in the winter of 2016/2017 (both $P > 0.05$). Likewise, the intensified frost increased NO_3^--N concentrations in both winters (F = 16.575, $P < 0.01$; Figure 7b). NO_3^--N concentrations were significantly higher in the treatment than the control plots in the ETP of 2015/2016 (t = 2.309, $P < 0.05$) and in the FP of 2016/2017 (t = 5.017, $P < 0.01$). Neither NH_4^+-N nor NO_3^--N concentration, however, differed between the frost regimes in the MGS of 2016 and 2017 (both $P > 0.05$).

3.6 Aggregate fraction and fine-root biomass

The relative distribution of the aggregate-size classes of the bulk soil was in the order small macroaggregates (0.25-2 mm) > large macroaggregates (>2 mm) > microaggregates (<0.25 mm) irrespective of frost regime (F = 221.75, $P < 0.001$; Table 2). The snow-exclusion treatment did not affect the distribution of aggregates in the size classes (F = 0.159, P = 0.897), the live fine-root biomass (F = 0.202, P = 0.663; Table 3) or the dead fine-root biomass (F = 0.171, P = 0.688) in the ETP.

4. Discussion

We investigated the impact of intensified soil frost on soil C cycling in a Tibetan subalpine spruce forest using a field snow-manipulation experiment. Our main objective was to determine whether differences in winter frost conditions induced immediate and carry-over effects on soil CO_2 efflux. Snow exclusion resulted in more intensive soil frost which, in line with the first hypothesis, decreased soil respiration in the winter season. However, contrary to what we expected, the increased winter frost did not have carry-over effects on soil respiration in the subsequent growing season. Several possible mechanisms may account for the underlying responses of CO_2 efflux during the snow-covered and snow-free seasons.

Firstly, due to the high thermal sensitivity of microbes, even small changes in soil temperature may shift their community structure and the ecological processes they drive (Davidson and Janssens, 2006; Schütt et al., 2014). Therefore, lower soil temperatures in the snow exclusion plots could potentially decrease winter CO_2 efflux. In our study, soil temperature only accounted for ~50% of the variance in winter CO_2 efflux. The temperature-CO_2 relationship was as weak as in temperate forests (Schindlbacher et al., 2007; Wang et al., 2010; Schindlbacher et al., 2014). In addition to soil temperature, other factors, such as the frequency of freeze-thaw cycles, soil moisture, and microbial activity, could also mediate winter soil respiration (Lipson et al., 2009; Du et al., 2013). Soil temperature alone therefore can not adequately explain the variation in winter CO_2 efflux.

We found high Q_{10} values in the winter regardless of the frost regime. Relatively high Q_{10} values of winter soil respiration have been also observed in temperate and boreal forests (Wang et al., 2010; Du et al., 2013). This can be explained by the fact that the winter soil CO_2 efflux was susceptible to small changes in soil temperature, particularly around the freezing mark (~ 0 °C). Significant changes were detected in soil CO_2 efflux over a narrow temperature span (~6 °C), resulting in high Q_{10} values in the winter. In addition, CO_2 pulses and microbial shifts associated with spring freeze-thaw cycles may further contribute to the observed result in the winter (Uchida et al. 2005; Wang et al., 2013). However, the relative importance of different factors warrants further investigation.

Other factors co-varying with soil temperature may also regulate winter CO_2 efflux. The intensified frost may have produced a stronger 'freezing drought', which could limit microbial activity and the extracellular diffusion of substrates (Rivkina et al., 2000). Soil moisture did not differ significantly between the treatment and control plots during the winters, suggesting that soil moisture was not likely responsible for the decreased winter CO_2 efflux. The lack of significant differences in CO_2 efflux during the snow-free seasons was likewise partially attributed to the lack of significant differences in both soil temperature and moisture between the treatment and control plots during the growing season.

Secondly, root activity is extremely low during dormant seasons, and winter soil respiration in cold ecosystems is primarily derived from microbial decomposition (Muhr et al., 2009; Wang et al., 2010). Winter soil respiration is thus largely determined by the biomass and activity of soil microbes (Lipson et al., 2002; Moorhead et al., 2014). Soil microbes are very susceptible to soil frost (Monson et al., 2006; Aanderud et al., 2013), which can kill a substantial proportion of the organisms by the rupture of cell membranes by ice crystals (Sulkava and Huhta, 2003; Jusselme et al., 2016; Gavazov et al., 2017). We also found that the increased frost significantly reduced soil microbial PLFAs, implying a lowered potential for the microbial community to metabolize soil C in the winters. A significant decline in the cross-winter microbial PLFAs also implied that seasonal frost would kill the soil microbes in the spruce forest irrespective of frost manipulation. Our measurements of microbial basal respiration, excluding plant roots, also indicated a similar decline with winter frost, consistent with *in situ* soil CO_2 efflux. Frost-induced decreases in winter soil respiration may thus largely be attributed to the lower microbial biomass and activity. Soil PLFAs and basal respiration in the middle of the growing season nevertheless did not differ significantly between frost regimes. These observations may partially account for the neutral effect of increased frost on CO_2 efflux during the snow-free growing seasons.

Soil enzymes play very important roles in the cycling of soil C and nutrients. Little attention has been paid to enzymatic activities in studies of winter climate change, despite the importance of soil enzymes in soil C cycling. A recent study found that enzymatic activities were negatively correlated with the intensity of soil frost in mixed-hardwood forests

(Sorensen et al., 2016), and another experiment also found that snow removal decreased the activity of soil invertase in an alpine spruce-fir forest (Tan et al., 2014). We assayed the activities of four enzymes involved in soil C cycling to further assess the functional capacity of soil. The intensified frost tended to reduce soil enzymatic activities. Soil enzymatic activities are strongly temperature-dependent (Tabatabai, 1982), so a decrease in soil temperature caused by snow-exclusion may, to some extent, reduce soil enzymatic activities directly. The lower activities may also partly be attributed to the smaller population size of the microbes, which are an important source of enzyme synthesis. Soil enzymes, as proximate agents of the decomposition of soil organic C, can break down plant and microbial cell walls and catabolize macromolecules into soluble substrates for microbial assimilation (Sinsabaugh et al., 2008). Frost-induced decreases in enzymatic activities may thus constrain this decomposition, which could also partly account for the lower winter CO_2 efflux. Conversely, intensified frost did not affect activities in the snow-free growing seasons, which may account for the lack of significant responses during the subsequent growing season.

Thirdly, soil frost may also have affected the decomposition of soil C in winter by altering nutrient availability. Intensified frost can increase the mortality of roots and microbes (Henry, 2007; Repo et al., 2014; Blume-Werry et al., 2016), which are important substrates for soil microbial metabolism during winter (Schimel et al., 2004). Dead roots and microbes are also main N sources during winter in cold systems (Chapin III et al., 1988; Tierney et al., 2001). In an earlier study we observed that soil at -5 °C could release considerable extractable N in the soils of this spruce forest, possibly due to the effect of freezing on microbial mortality (Xu et al., 2014). The snow-exclusion treatment in the present study stimulated the production of soil extractable N in the two winters, likely due mainly to the increased microbial mortality. An increase in N availability but a decrease in soil PLFAs throughout the winter, irrespective of the frost regime, may also support this conclusion. Live and dead fine-root biomass did not differ significantly between frost regimes later in the winter, further suggesting that the increased N availability was mainly attributable to microbial mortality rather than to root injury. An increase in N availability coincided with a decrease in CO_2 efflux, implying that the cycling of soil C could be decoupled from N availability during winter under intensified frost.

In addition to microbial and root mortality, substrate availability could have been affected by the physically disruptive effects of frost on soil aggregates (Chai et al., 2014). Freezing can break down macroaggregates into microaggregates (Oztas and Fayetorbay, 2003). Microaggregates with a larger surface area have more contact points, which can potentially increase the amount of substrate decomposed by microorganisms (Grogan et al., 2004). Snow removal increased the fraction of microaggregates in a northern hardwood forest, implying that soil substrate could become more accessible to soil microorganisms (Steinweg et al., 2008). Our observations, however, did not provide further evidence that more intense frost could disrupt aggregates in the soil of this Tibetan spruce forest. The intensified frost did not affect the distribution of aggregates among the size classes, suggesting that frost-associated changes to aggregates may not importantly affect soil respiration in the spruce forest during the winter and growing season.

Lastly, the flux of CO_2 derived from decaying litter accounts for a considerable part of total soil respiration during winter (Uchida et al., 2005). CO_2 flux derived from aboveground litter accounts for an average of 14.2% of total soil respiration in this spruce-forest stand (Xiong et al., 2015). In a previous study we also found that the mass loss of spruce needles over the winter constituted 18.3-28.8% of the net loss rates for the entire year (Xu et al., 2016). The lack of snow cover at this experimental site decreased the temperature of the surface soil by an average of 1.4 °C during the winter (Li et al., 2017), implying that litter decomposition was most likely inhibited by the lower temperatures. A growing number of studies have documented that thick snow covers can provide relatively stable conditions for biological activity, favoring the decomposition of plant litter (Christenson et al., 2010; Bokhorst et al., 2013; Saccone et al., 2013). The rates of decomposition of litter from subalpine tree species in this area similarly decrease with decreasing snow depth (Ni et al., 2014; He et al., 2015). The lower rate of litter decomposition due to the lack of snow cover may therefore also have contributed to the lower winter soil respiration in the snow-exclusion plots, but further supporting evidence is needed.

5. Implications

The climate on the Tibetan Plateau has changed considerably in recent decades, especially in winter (Wang et al., 2016). Winter snowfall has tended to decrease substantially due to strong winter warming (Xu et al., 2017). The decrease or absence of insulating snow cover associated with climate change may thus increase the duration and intensity of soil frost in the future in this special region. The importance of soil frost, large storage of soil C and sensitivity of snow cover to winter warming indicate that understanding the potential effects of projected frost increases on soil C cycling in the subalpine forests of western China is essential. To our knowledge, our study is the first to identify the effects of changes in soil frost on soil C cycling in a Tibetan forest. Our results generally indicate that more intense soil frost decreases winter soil respiration and biological activities. Winter soil CO_2 emission was lower in the snow-exclusion than in the control plots during the two winters of the study. Intensified soil frost, however, did not affect soil CO_2 efflux and biological activities during the subsequent growing season, suggesting that a short-term change in snow cover does not produce large carry-over effects in snow-free periods. If the observed effects apply to natural conditions, intensified soil frost would decrease the amount of soil C released to the atmosphere from subalpine forests during winter, but additional supporting evidence is needed.

This study was conducted during two contrasting winters (cold winter and thick snow cover in 2015/2016 and mild winter and thin snow cover in 2016/2017) so offered a good opportunity for determining the effect of the lack of snow cover in winters with different weather on soil C cycling in the Tibetan spruce forest. The decrease in soil respiration due to frost in the first winter occurred early but then disappeared, suggesting that soil biological processes may begin to acclimate to the frost late in the winter. Soil respiration early in the mild winter of 2016/2017 did not differ significantly between the treatment and control plots, mainly due to the absence of an insulating snow cover. CO_2 effluxes, however, were lower in the treatment plots after the formation of a steady snow cover (>10 cm). In addition to variable snowfall, extreme winter events (e.g. warm weather and snow storms) may become more frequent and likely under scenarios of future climate, indicating the complexity and uncertainty of winter climate change in this specific region. The comparably strong climate change and variable winter snowfall on the Tibetan Plateau bring great challenges and opportunities for studying winter climate change and its impacts on the structure and function of Tibetan ecosystems. Long-term monitoring is strongly needed for exploring the natural winter variations and underlying mechanisms of the observed phenomena to help developing models for providing more realistic predictions of future winter conditions.

The frost intensity due to the lack of snow cover was low at our experimental forest site, unlike in temperate and boreal forests (e.g. Groffman et al., 2006; Muhr et al., 2009; Sorensen et al., 2016), but the difference in temperature minima was nearly 2 °C, likely due to the site-specific characteristics, such as winter snowfall, air temperature, properties of soil heat transfer and albedo. Such soil frost, however, had large impacts on soil respiration, microbial PLAFs, enzymatic activity and N availability, suggesting that Tibetan forest soils will be sensitive to changing soil frost in the future. The direction and magnitude of the response of soil respiration to intensified soil frost may largely depend on the interaction between less snowfall and warmer temperature in winter. Winter warming may offset the negative effects induced by frost to some extent. Seasonal snow cover in cold regions plays a key role in decoupling soil from cold winter weather, but soil temperature is often insensitive to a small change in air temperature. Changes in snow cover will thus likely have a stronger influence on soil biological processes than winter warming itself (Groffman et al., 2001a), and long-term changes in soil frost may also have carry-over effects on soil C dynamics during the subsequent growing season in cold systems (Zhao et al., 2017). More research is warranted to integrate potential factors and separate their relative importance for a better understanding and ability to predict potential C feedbacks in snowy regions under a warmer future.

6. Conclusions

This study explored the immediate and carry-over effects of intensified soil frost on soil C cycling in a subalpine spruce forest on the Tibetan Plateau of China. Our results suggested that a lack of snow cover increase the intensity of soil frost, which decrease soil respiration in the winter; whereas we did not find any legacy effects during the subsequent growing seasons. Frost decreased microbial biomass and activity in the winter but not in the snow-free growing season. More intense soil frost did not affect the size distribution of soil aggregates or fine-root biomass. Predicted increase in soil frost driven by winter climate change may, therefore, decrease winter soil respiration by direct environmental effects (e.g. soil temperatures) and by indirect effects on soil biological properties (e.g. microbial biomass and activities) in subalpine forests on the Tibetan Plateau. Further, intensified soil frost did not cause cross-seasonal effects on soil CO_2 efflux and microbial activity in the subsequent growing seasons. Our results highlight the ecological importance of a continuous seasonal snowpack and microbe-associated C processes in subalpine forest ecosystems. These findings improve our understanding of the response of soil C dynamics to winter climate change in this region experiencing large decreases in winter snowfall.

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Competing interests

The authors declare no competing financial interests.

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Table 1 Mean soil CO_2 efflux (μmol m^{-2} s^{-1}, means ± SEs) during the winter and growing season.

Year	Period	Treatment	Control
2016	Winter	0.47 ± 0.08	0.55 ± 0.08
	Growing season	2.07 ± 0.28	2.19 ± 0.29
2017	Winter	0.43 ± 0.06	0.53 ± 0.07
	Growing season	2.23 ± 0.26	2.25 ± 0.30

Table 2. Relative distribution of the aggregate size classes (%, means ± SEs) in the snow-exclusion treatment and control plots in the early thawing period of 2016 and 2017.

Year	Size	Treatment	Control
2016	>2 mm	34.9 ± 5.7	32.9 ± 6.9
	0.25-2 mm	59.9 ± 4.7	58.6 ± 4.2
	<0.25 mm	5.2 ± 2.0	8.5 ± 2.8
2017	>2 mm	35.3 ± 6.4	36.7 ± 4.5
	0.25-2 mm	53.9 ± 3.8	50.7 ± 2.2

	<0.25 mm	10.8 ± 3.6	12.6 ± 2.8

Table 3. Content of live and dead fine roots (g m-2, means ± SEs) up to a depth of 15 cm in the snow-exclusion treatment and control plots in the early thawing period of 2016 and 2017.

Year	Fine root pool	Treatment	Control
2016	Live	243.7 ± 45.6	251.7 ± 35.6
	Dead	92.8 ± 14.4	79.4 ± 26.3
2017	Live	221.2 ± 38.5	215.6 ± 28.9
	Dead	94.3 ± 22.6	88.1 ± 16.4

Figure legends

Figure 1. Air temperature, soil temperature and snow depth (a) and soil moisture (b) in the snow-exclusion treatment and control plots during the experimental period.

Figure 2. Soil CO_2 efflux (means ± SEs) in the snow-exclusion treatment and control plots during the experimental period. Significant differences between the control and treatment on a given date are indicated by asterisks ($P < 0.05$).

Figure 3. Exponential relationships between soil CO_2 efflux and soil temperature (means ± SEs) in the treatment and control plots for winter, growing season and entire year, respectively.

Figure 4. Total soil phospholipid fatty acids (means ± SEs) in the snow-exclusion treatment and control plots. Significant differences between the control and treatment on a given date are indicated by asterisks ($P < 0.05$). FP, frost period; ETP, early thawing period; MGS, middle of the growing season.

Figure 5. Soil microbial respiration (means ± SEs) in the snow-exclusion treatment and control plots. Significant differences between the control and treatment on a given date are indicated by asterisks ($P < 0.05$). FP, frost period; ETP, early thawing period; MGS, middle of the growing season.

Figure 6. Activities of (a) β-glucosidase, (b) polyphenol oxidase, (c) peroxidase and (d) β-N-acetyl-glucosaminidase (means ± SEs) in the snow-exclusion treatment and control plots. Significant differences between the control and treatment on a given date are indicated by asterisks ($P < 0.05$). FP, frost period; ETP, early thawing period; MGS, middle of the growing season.

Figure 7. Soil ammonium (a) and nitrate (b) concentrations (means ± SEs) in the

snow-exclusion treatment and control plots. Significant differences between the control and treatment on a given date are indicated by asterisks ($P < 0.05$). FP, frost period; ETP, early thawing period; MGS, middle of the growing season.

Figure 1

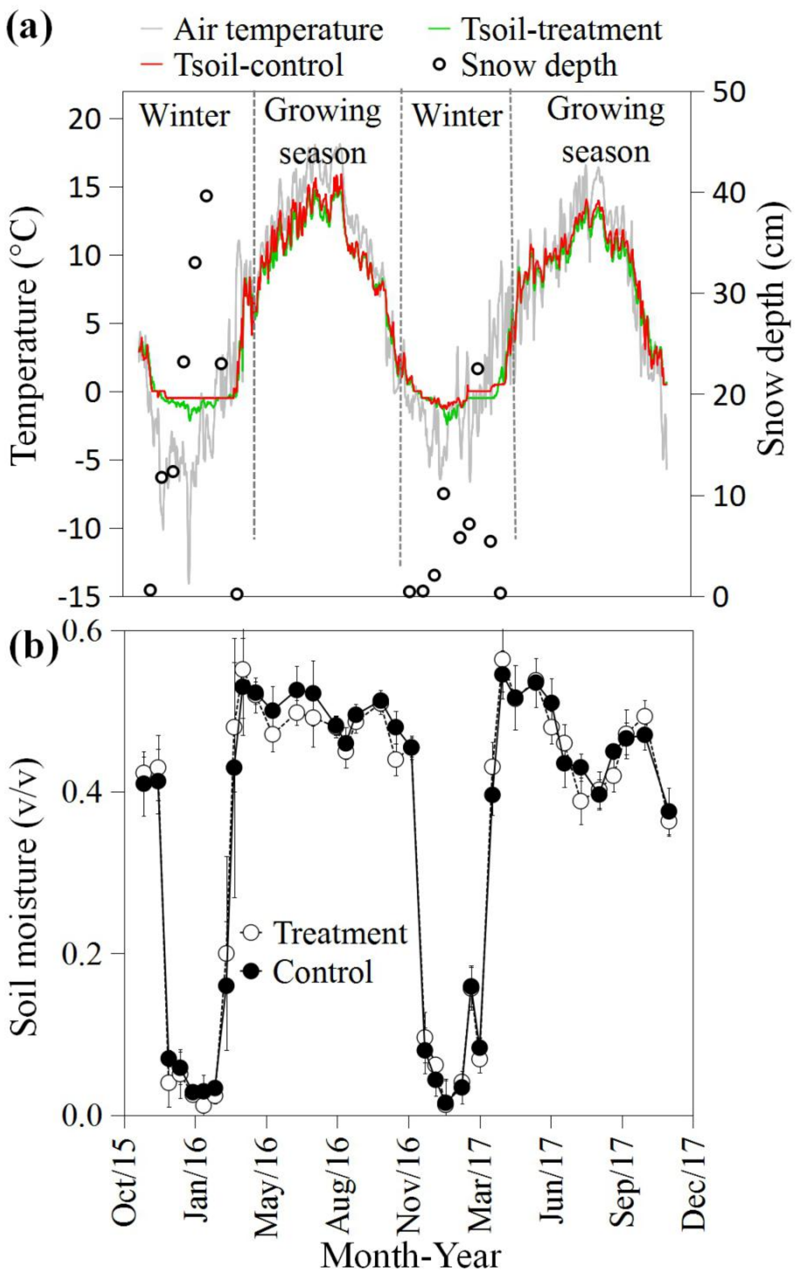


Figure 2

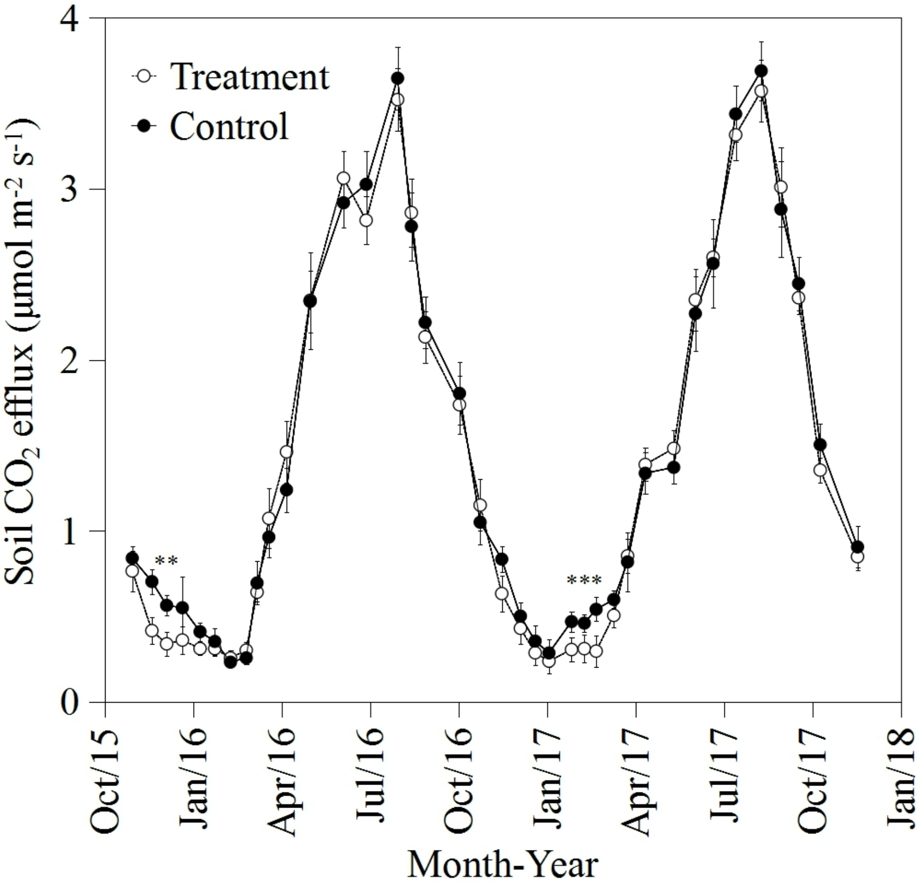

4
3
2
1
0
Treatment
Control
Soil CO$_2$ efflux (µmol m^{-2} s^{-1})
**
***
Oct/15
Jan/16
Apr/16
Jul/16
Oct/16
Jan/17
Apr/17
Jul/17
Oct/17
Jan/18
Month-Year

Figure 3

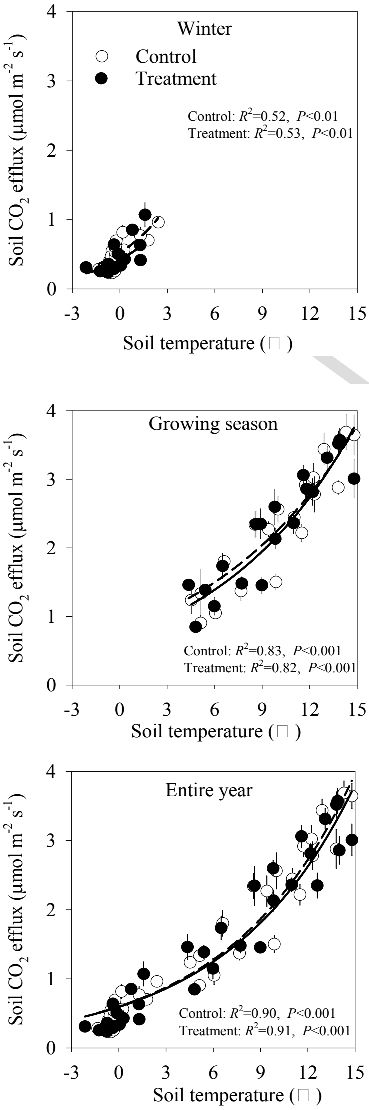

Winter
○ Control
● Treatment
Control: R^2=0.52, P<0.01
Treatment: R^2=0.53, P<0.01
Soil CO$_2$ efflux (μmol m^{-2} s^{-1})
Soil temperature (□)
Growing season
Control: R^2=0.83, P<0.001
Treatment: R^2=0.82, P<0.001
Entire year
Control: R^2=0.90, P<0.001
Treatment: R^2=0.91, P<0.001
0
1
2
3
4
-3
0
3
6
9
12
15

Figure 4

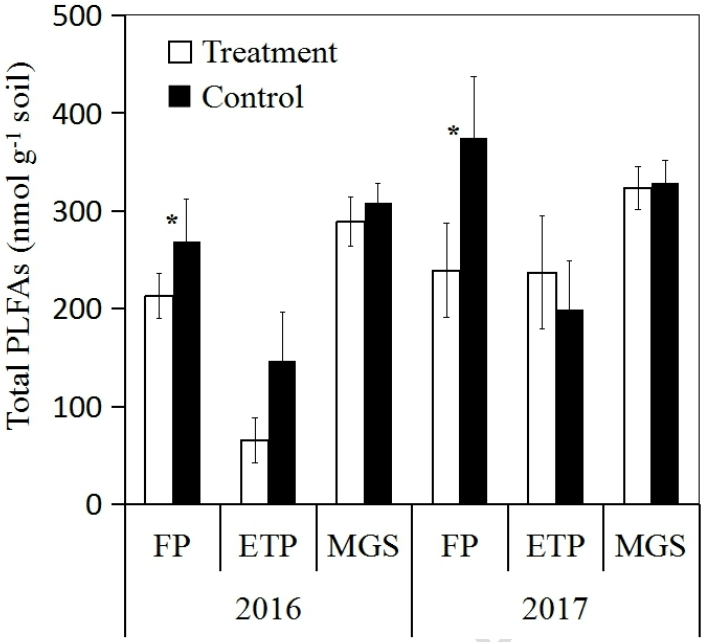

500
400
300
200
100
0
Total PLFAs (nmol g^{-1} soil)
□ Treatment
■ Control
*
*
FP
ETP
MGS
FP
ETP
MGS
2016
2017

Figure 5

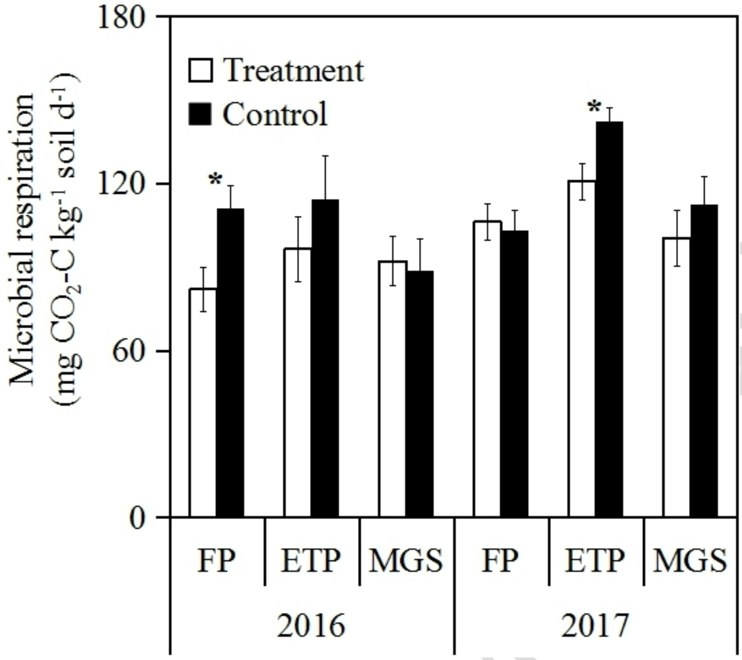

Microbial respiration
(mg CO₂-C kg⁻¹ soil d⁻¹)
180
120
60
0
☐ Treatment
■ Control
*
*
FP
ETP
MGS
FP
ETP
MGS
2016
2017

Figure 6

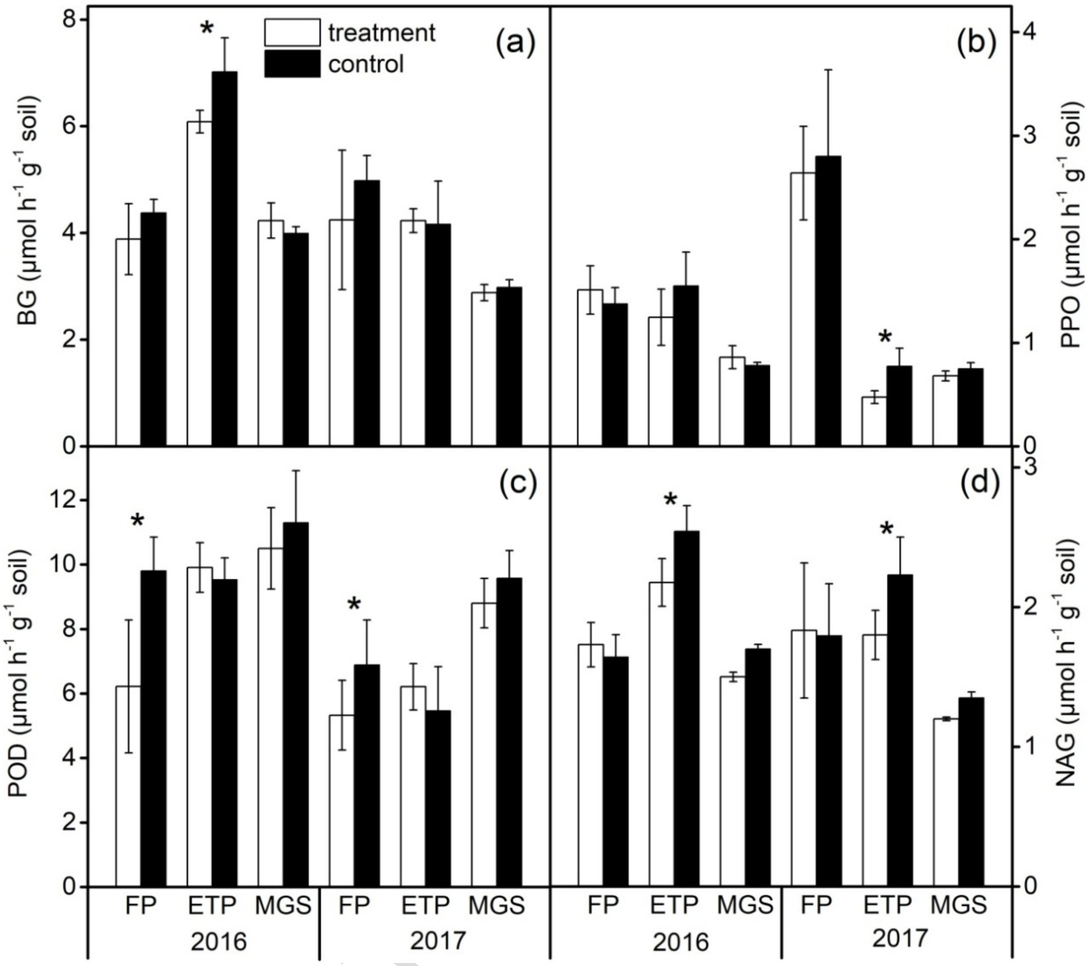
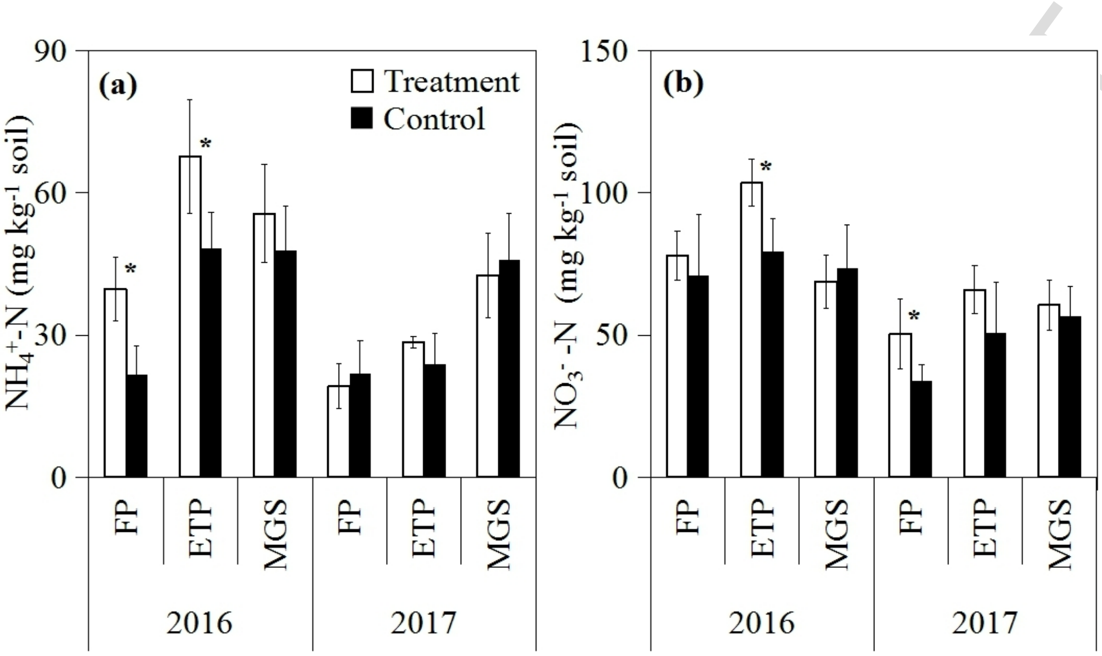

(a)
(b)
(c)
(d)
treatment
control
BG (μmol h^{-1} g^{-1} soil)
PPO (μmol h^{-1} g^{-1} soil)
POD (μmol h^{-1} g^{-1} soil)
NAG (μmol h^{-1} g^{-1} soil)
0
2
4
6
8
10
12
1
3
*
FP
ETP
MGS
2016
2017

Figure 7


(a)
□ Treatment
■ Control
NH_4^+-N (mg kg^{-1} soil)
90
60
30
0
*
*
FP
ETP
MGS
FP
ETP
MGS
2016
2017
(b)
NO_3^--N (mg kg^{-1} soil)
150
100
50
0
*
*
FP
ETP
MGS
FP
ETP
MGS
2016
2017

Highlights

- Increased frost reduced soil microbial biomass and enzyme activity in winter.
- Increased frost reduced soil respiration in winter.
- Increased frost unaffected biological processes in the snow-free growing season.